Supporting Information to:

Determination of urinary metabolites of the emerging UV filter Octocrylene by online-SPE-LC-MS/MS

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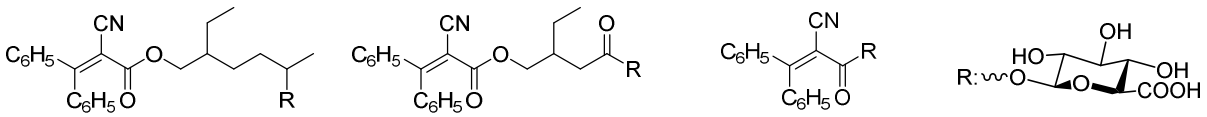
This Supporting Information includes:

Structural formulae of glucuronides; reagents and materials; syntheses and characterization of products; further details on HR-MS and UHR-MS conditions, preparation of standard solutions, chromatographic conditions, estimation of LOQs, and discussion of enzymatic hydrolysis and MS/MS fragmentation behavior of analytical standards; exemplary calibration curves.

Structural formulae of glucuronides



5OH-OC glucuronide (553.6 g/mol) | DOCCA glucuronide (539.5 g/mol) | CPAA glucuronide (425.4 g/mol)

Figure S-1. Structural formulae of glucuronides of the investigated OC metabolites, hydrolyzed during sample preparation

Supporting information to Experimental Section

Reagents and Materials

Ethyl 3,3-diphenyl-2-cyanoacrylate, d_{10}-benzophenone, liquid ammonia (lecture bottle), ethyl cyanoacetate, 2(5*H*)-furanone, ethyl magnesium chloride (EtMgCl, 2 M in THF), trimethylsilyl chloride (TMS-Cl), allyl bromide, copper(I) bromide - dimethyl sulfide complex (CuBr*Me_2S), tetrakis(triphenylphosphine)palladium (0) [$(Ph_3P)_4Pd$], *N,N*'-dicyclohexyl carbodiimide (DCC), 4-dimethylaminopyridine (DMAP), Titanium tetrachloride ($TiCl_4$, 1 M in toluene), acetic acid puriss./Ph. Eur., formic acid p.a., and ammonium acetate BioXtra ⩾98% were purchased from Sigma-Aldrich (Steinheim, Germany). 2-Cyano-3,3-diphenylacrylic acid (CPAA) was synthesized as described by Li et al 2013[49]. Acetonitrile, methanol and water CHROMASOLV^{TM} LC-MS grade were obtained from Honeywell Riedel-de Haën (Seelze, Germany). Ultrapure water was produced by an in-house Advantage A10 water purification unit (Merck Millipore, Darmstadt, Germany). β-glucuronidase from E. coli K12 (at least 140 u/mL at 37° C according to manufacturer) was obtained from Roche Diagnostics (Mannheim, Germany). For LC-MS analysis silanized HPLC vials (Macherey-Nagel, Düren, Germany) with silicone/PTFE screw caps (VWR, Leuven, Belgium) were used.

Syntheses of Analytical Standards

2-Cyano-3,3-d_{10}-diphenylacrylic acid (CPAA-d_{10}). d_{10}-Benzophenone imine was obtained from d_{10}-benzophenone and gaseous ammonia in toluene, in the presence of $TiCl_4$.[51] d_{10}-Benzophenone imine (0.60 g, 3.1 mmol) was heated with ethyl cyanoacetate (0.50 g, 4.4 mmol) in toluene (10 mL) at reflux (30 min), then toluene was distilled off, and the residue was heated at 160 °C (bath temp.) for 30 min. Flash chromatography on regular SiO_2 (25 g) with gradient of ethyl acetate in cyclohexane (10 – 50%) afforded ethyl 2-cyano-3,3-d_{10}-diphenylacrylate (0.6 g, 68%) and ca. 60 mg of d_{10}-benzophenone (impurity in d_{10}-benzophenone imine). Ethyl 2-cyano-3,3-d_{10}-diphenylacrylate (0.6 g, 2.1 mmol) was saponified with NaOH in aq. ethanol as described for the unlabeled compound;[49] CPAA-d_{10} (400 mg, 74%) was isolated by chromatography on SiO_2 (25 g, application on Celite, gradient of ethyl acetate in cyclohexane, 33% - 80%).

2-Ethyl-5-hydroxyhexyl 2-cyano-3,3-diphenylacrylate (5OH-OC) and 2-Ethyl-5-hydroxyhexyl 2-cyano-3,3-d_{10}-diphenylacrylate (5OH-OC-d_{10}). 2-Ethylhexane-1,5-diol[52] (1:1 mixture of 2 diastereomers, 150 mg, 1.0 mmol) and CPAA (180 mg, 0.72 mmol) or CPAA-d_{10} (180 mg, 0.69 mmol) were dissolved in anhydrous CH_2Cl_2 (10 mL; the acids dissolved partially), and then 4-(dimethylamino)pyridine (DMAP) (12 mg, 0,1 mmol) was added to each reaction mixture. The reaction mixtures were stirred and cooled in an ice bath, and the solutions of *N,N*'-dicyclohexyl carbodiimide (DCC) (206 mg, 1.0 mmol) in CH_2Cl_2 (1.0 mL) were added dropwise via syringe within 20 min. The acids dissolved gradually. The reaction mixtures were allowed to warm-up and stirred at room temperature overnight, concentrated, taken-up in pentane – diethyl ether mixture (1:1, 10 mL), and the precipitates (*N,N'*-dicyclohexylurea) were removed by filtration. The filtrates were applied onto columns with SiO_2 (50 g). Elution with a gradient of diethyl ether in pentane (50 – 100%) afforded title compounds (~190 mg, 70%).

Dihydro-4-ethyl-2(3*H*)-furanone was synthesized from 2(5*H*)-furanone according to the method described for an *n*-propyl analog.[53] Copper(I) bromide dimethyl sulfide complex (5.0 g, 24 mmol) was suspended in anhydrous diethyl ether (30 mL) at -20°C, EtMgCl (24 mL of 2 M solution in THF, 48 mmol) was added at -20°C, and the slurry was stirred at -10°C for 15 min. Then 2(5*H*)-furanone (1.0 g, 12 mmol) and chlorotrimethylsilane (1.52 g, 1.77 mL, 14 mmol) were added to the reaction mixture dropwise simultaneously from two syringes at -10°C in the time course of 15 – 20 min. The reaction mixture was allowed to warm-up to rt, stirred for 30 min at rt, cooled to 0°C, and then sat. aq. solution of NH_4Cl (30 mL) was added carefully. The aqueous layer was separated and extracted with ether (2×50 mL). Combined organic solutions were dried for 2 d over anhydrous Na_2SO_4 with addition of tetrabutylammonium fluoride trihydrate (4.4 g, 14 mmol). After filtration and rinsing the precipitate with ether, combined organic solutions were evaporated in vacuo (bath temp. 45°C, 550 mbar). Yield of the crude title compound was 0.89 g (65%).

Allyl 3-(hydroxymethyl)pentanoate. Crude dihydro-4-ethyl-2(3*H*)-furanone (0.89 g, 7.8 mmol) was vigorously stirred with 1 M aq. NaOH (8.0 mL, 8.0 mmol) overnight at rt. The reaction mixture was extracted with ether (2 x 5 mL), the aqueous layer was frozen and freeze-dried. The semi-solid residue was dissolved in water (20 mL), solid CO_2 was carefully added in small portions, until pH-value reached 8-9, the solution was frozen and lyophilized once more. The residue was suspended in dry DMF (10 mL), allyl bromide (1.82 g, 1.30 mL, 15 mmol) was added, and the reaction mixture was vigorously stirred at 50°C overnight. Volatile materials (allyl bromide and DMF) were evaporated in vacuo, and the residue was applied onto a column with 60 g SiO_2. Elution with hexane – ethyl acetate (1:1) afforded the title compound (0.76 g, 56%).

2-(Carboxymethyl)butyl 2-cyano-3,3-diphenylacrylate (DOCCA) and 2-(carboxymethyl)butyl 2-cyano-d_{10}-3,3-diphenylacrylate (DOCCA-d_{10}). CPAA (283 mg, 1.15 mmol) or CPAA-d_{10} (290 mg, 1.12 mmol), each was combined with allyl 3-(hydroxymethyl)pentanoate (180 mg, 1.14 mmol) in anhydrous CH_2Cl_2 (10 mL; the acids dissolved partially), and then DMAP (12 mg, 0,1 mmol) was added to each reaction mixture. The reaction mixtures were stirred and cooled in an ice bath, and the solutions of DCC (235 mg, 1.14 mmol) in CH_2Cl_2 (1.5 mL) were added dropwise via syringe within 20 – 25 min. The acids dissolved gradually. The reaction mixtures were allowed to warm-up and stirred at rt overnight, concentrated, taken-up in pentane – diethyl ether mixture (1:1, 10 mL), and the precipitates (*N,N'*-dicyclohexylurea) were removed by filtration. The filtrates were applied onto columns with SiO_2 (50 g). Elution with a gradient of ethyl acetate in hexane (1:1 – 1:0) afforded **2-(allyloxycarbonylmethyl)butyl 3,3-diphenyl-2-cyanoacrylate** (400 mg) **and 2-(carboxymethyl)butyl 2-cyano-d_{10}-3,3-diphenylacrylate** (360 mg) as oils. They contained traces of *N*-acylureas formed from DCC and CPAA (CPAA-d_{10}) and were used in the final deprotection steps without further characterization. Allyl esters were dissolved in anhydrous acetonitrile (5.0 mL), and HCOOH (0.3 mL), Et_3N (0.6 mL) and $Pd(Ph_3P)_4$ (40 mg, ~4 %mol) were added successively to each solution under argon at 0°C. After warming up to room temperature, the reaction mixture was stirred for 2 h. TLC displayed that the starting allyl esters were consumed, and new substances with lower R_f were formed (hexane – ethyl acetate, 1:3, UV detection at 254 nm). The reaction mixtures were diluted with diethyl ether (50 mL each), washed with 1 M aq. $KHSO_4$ (20 mL each), brine (20 ml each) and dried over anhydrous Na_2SO_4. After filtration and concentration *in vacuo*, the title acids – **DOCCA** (235 mg, yield 57% over 2 steps) and **DOCCA-d_{10}** (100 mg, yield 24% over 2 steps) – were isolated by chromatography on SiO_2 (25 g) using gradient of ethyl acetate (with 2%v/v of HCOOH, 10 – 50%) in cyclohexane.

Characterization of synthesis products

2-Cyano-3,3-diphenylacrylic acid (CPAA): HPLC (Kinetex C18, 50 × 3 mm, 2.6 µm, water – acetonitrile, both with 0.1%v/v TFA, flow rate: 0.25 mL/min, gradient: 20 – 95% acetonitrile in 10 min, detection at 254 nm): t_R = 8.4 min (peak area 95.3%), 9.6 min (4.7%).

Ethyl 3,3-*d*$_{10}$-diphenyl-2-cyanoacrylate: colorless solid, ^{1}H NMR (400 MHz, CD_3CN; figure S-2) δ 4.12 (q, *J*=7 Hz, 2H), 1.08 (t, *J*=7 Hz, 3H).

2-Cyano-3,3-*d*$_{10}$-diphenylacrylic acid (CPAA-*d*$_{10}$): colorless solid with m. p. 210 °C, HPLC (Kinetex C18, 50 × 3 mm, 2.6 µm, water – acetonitrile, both with 0.1%v/v TFA, flow rate: 0.25 mL/min, gradient: 20 – 95% acetonitrile in 10 min, detection at 254 nm): t_R = 8.2 min (peak area 99.1%), 9.4 min (0.9%). HR-MS (ESI, positive ion mode): *m/z* 282.1311 (+0.4 ppm) $[M+Na]^+$.

2-Ethyl-5-hydroxyhexyl 2-cyano-3,3-diphenylacrylate (5OH-OC): oil, HPLC (Eurospher C18, 250 × 4 mm, 5 µm, water – acetonitrile, both with 0.1%v/v TFA, flow rate: 1.1 mL/min, gradient: 30 – 100% acetonitrile in 25 min, detection at 254 nm): t_R = 20.5 min (peak area 100%). ^{1}H NMR (400 MHz, CD_3CN, 1:1 mixture of 2 diastereomers; figure S-3) δ 7.61 – 7.34 (m, 8H), 7.18 (m, 2H), 4.01 (m, 2H, CH_2O), 3.61 (m, 1H, C<u>H</u>OH), 2.51 (dd, *J* = 4.8 and 2.8 Hz, OH, 1H), 1.41 (m, 1H), 1.32 and 1.21 (m×2, Σ 6H), 1.09 (2xd, *J* = 6.2 Hz, 3H), 0.82 (2xt, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, CD_3CN, 1:1 mixture of 2 diastereomers; figure S-4) δ 170.3, 164.0, 140.3/140.2, 132.6 (CH), 131.6 (CH), 131.2 (CH), 130.5 (CH), 129.9 (CH), 129.7 (CH), 118.3, 69.4 (CH_2), 68.5/68.4 (CH), 40.0/39.9 (CH), 37.5/37.4 (CH_2), 27.8/27.7 (CH_2), 24.6 (CH_2), 24.26/24.29 (CH_3), 11.66/11.67 (CH_3). HR-MS (ESI, positive ion mode): *m/z* 400.1884 (+0.3 ppm) $[M+Na]^+$.

2-Ethyl-5-hydroxyhexyl 2-cyano-3,3-*d*$_{10}$-diphenylacrylate (5OH-OC-*d*$_{10}$): oil, HPLC (Interchim (France) C18, 250 × 4.6 mm, 10 µm, water – acetonitrile, both with 0.1%v/v TFA, flow rate: 1.0 mL/min, gradient: 50% acetonitrile, 0 – 3 min, 50 – 100% acetonitrile in 15 min, detection at 254 nm): t_R = 7.3 min (peak area 100%). ^{1}H NMR (400 MHz, CD_3CN, 1:1 mixture

of 2 diastereomers; figure S-5) δ 4.01 (m, 2H, CH_2O), 3.61 (m, 1H, C<u>H</u>OH), 1.41 (m, 1H), 1.30 and 1.20 (m×2, Σ 6H), 1.09 (2xd, *J* = 6.2 Hz, 3H), 0.82 (2xt, *J* = 7.4 Hz, 3H). HR-MS (ESI, positive ion mode): *m/z* 410.2510 (-0.2 ppm) $[M+Na]^+$.

Dihydro-4-ethyl-2(3*H*)-furanone: relatively volatile light oil; polymerizes by heating, ^{1}H NMR (400 MHz, $CDCl_3$; figure S-6) δ 4.41 (dd, *J* = 9.0 and 7.4 Hz, 1H), 3.94 (dd, *J* = 9.0 and 7.0 Hz, 1H), 2.62 (dd, *J* = 17.2 and 8.4 Hz, 1H), 2.52 – 2.42 (m, 1H), 2.18 (dd, *J* = 17.2 and 7.8 Hz, 1H), 1.51 (m, *J* = 7.4 and 3.1 Hz, 2H), 0.95 (t, *J* = 7.4 Hz, 3H).

Allyl 3-(hydroxymethyl)pentanoate: oil, ^{1}H NMR (400 MHz, $CDCl_3$; figure S-7) δ 5.92 (ddt, *J* = 17.2, 10.4 and 5.8 Hz, 1H), 5.32 (dq, *J* = 17.2 and 1.5 Hz, 1H), 5.23 (dd, *J* = 10.4 and 1.3 Hz, 1H), 4.58 (dt, *J* = 5.8 and 1.4 Hz, 2H), 3.66 (dd, *J* = 10.9 and 4.7 Hz, 1H), 3.52 (dd, *J* = 11.0 and 6.7 Hz, 1H), 2.49 – 2.33 (m, 2H), 1.97 (m, *J* = 7.2, 6.7, 5.7 and 4.6 Hz, 1H), 1.52 – 1.24 (m, 2H), 0.93 (t, *J* = 7.5 Hz, 3H).

2-(Carboxymethyl)butyl 2-cyano-3,3-diphenylacrylate (DOCCA): colorless solid with m. p. 179 °C, HPLC (Eurospher C18, 250 × 4 mm, 5 µm, water – acetonitrile, both with 0.1%v/v TFA, flow rate: 1.1 mL/min, gradient: 50 – 100% acetonitrile in 25 min, detection at 254 nm): t_R = 10.2 min (peak area 98%). ^{1}H NMR (400 MHz, $CDCl_3$; figure S-8) δ 7.54 – 7.31 (m, 8H), 7.18 (m, 2H), 4.15 (dd, *J* = 11.7 and 4.7 Hz, 1H), 4.02 (dd, *J* = 11.1 and 6.3 Hz, 1H), 2.20 (d, *J* = 6.7 Hz, 2H), 2.01 (m, *J* = 6.7 and 4.8 Hz, 1H), 1.30 (m, 2H), 0.87 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$; figure S-9 and S-10) δ 178.3, 169.6, 162.7, 138.6, 138.3, 131.6 (CH), 130.5 (CH), 130.3 (CH), 129.3 (CH), 128.5 (CH), 128.4 (CH), 116.7, 103.6, 67.7 (OCH_2), 35.7 (CH), 35.5 (CH_2), 23.7 (CH_2), 11.2 (CH_3). HR-MS (ESI, positive ion mode): *m/z* 386.1363 (±0.0 ppm) $[M+Na]^+$.

2-(Carboxymethyl)butyl 2-cyano-d_{10}-3,3-diphenylacrylate (DOCCA-d_{10}): colorless solid, HPLC (Eurospher C18, 250 × 4 mm, 5 µm, water – acetonitrile, both with 0.1%v/v TFA, flow

rate: 1.1 mL/min, gradient: 50 – 100% acetonitrile in 25 min, detection at 254 nm): t_R = 10.2 min (peak area 99%). ^{1}H NMR (400 MHz, $CDCl_3$; figure S-11) δ 4.17 (dd, *J* = 11.1 and 4.8 Hz, 1H), 4.03 (dd, *J* = 11.1 and 6.4 Hz, 1H), 2.22 (dd, *J* = 6.8 and 1.7 Hz, 2H), 2.01 (m, *J* = 6.7 and 4.7 Hz, 1H), 1.31 (m, J = 7.8 and 6.8 Hz, 2H), 0.88 (t, *J* = 7.5 Hz, 3H), HR-MS (ESI, positive ion mode): *m/z* 396.1991 (±0.0 ppm) $[M+Na]^+$.

^{1}H NMR (400 MHz, Acetonitrile-d_3) δ 4.17 – 4.07 (m, 2H), 1.12 – 1.04 (m, 3H).

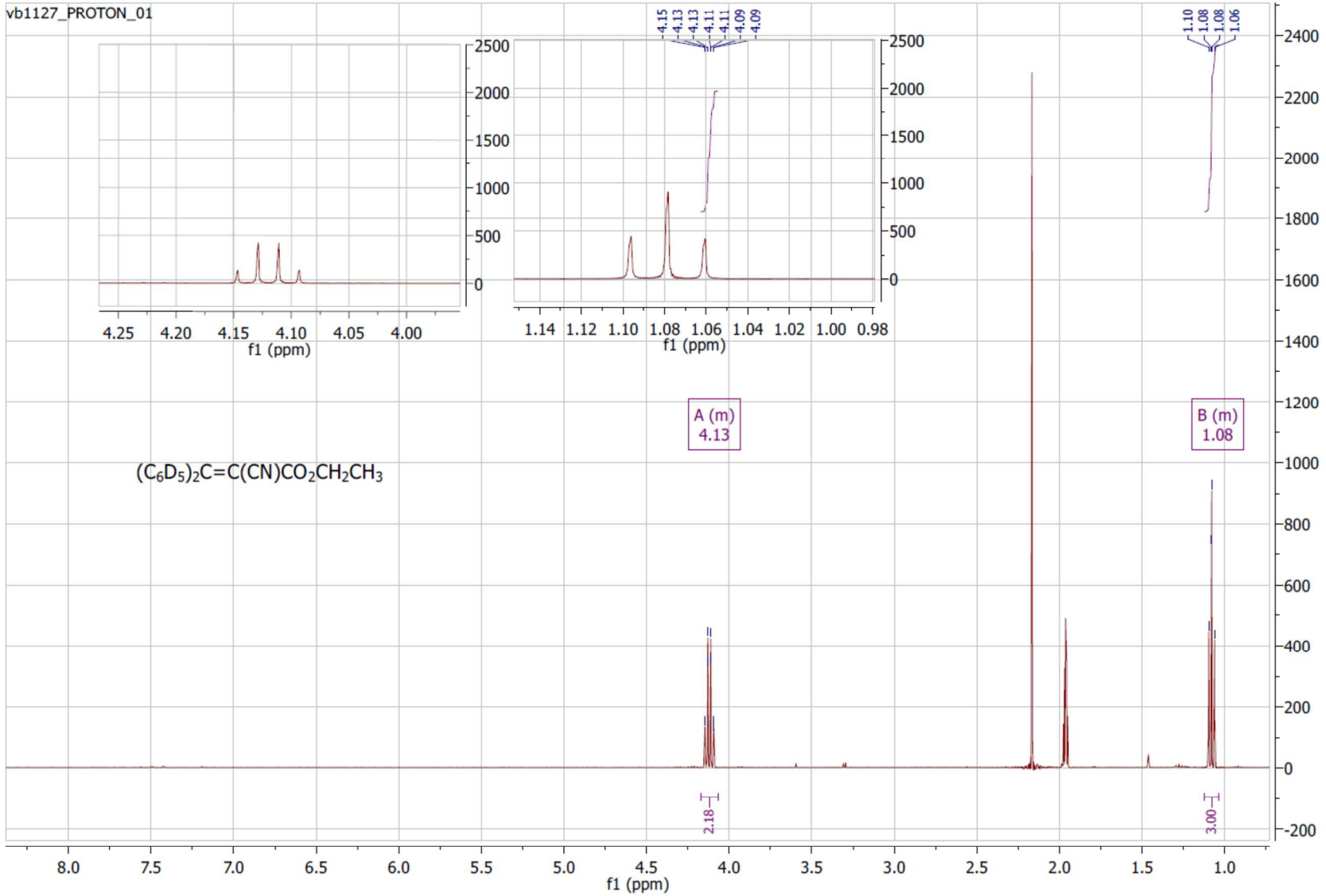


Figure S-2. ^{1}H NMR spectrum of ethyl 3,3-d_{10}-diphenyl-2-cyanoacrylate

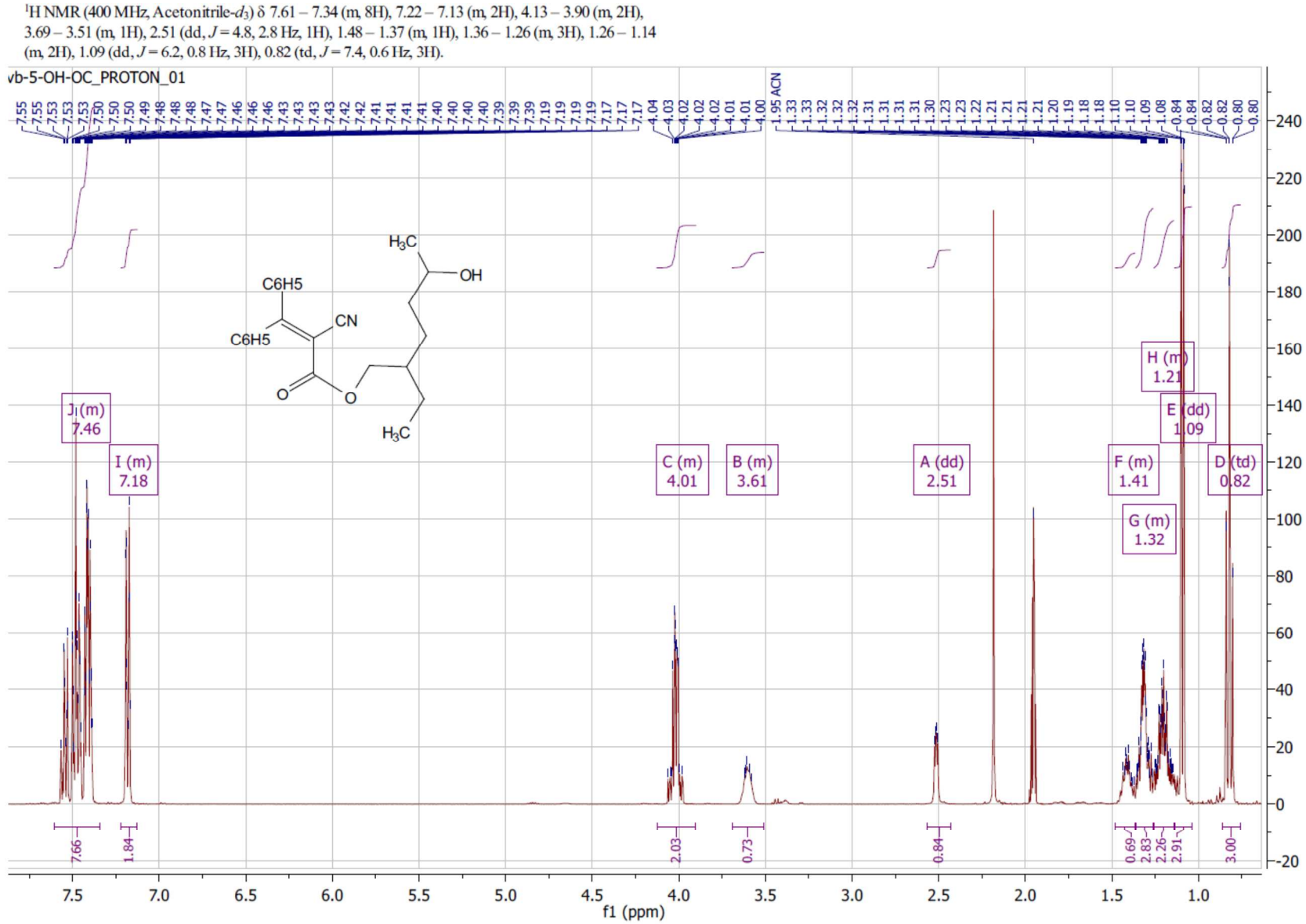


Figure S-3. ^{1}H NMR spectrum of 5OH-OC

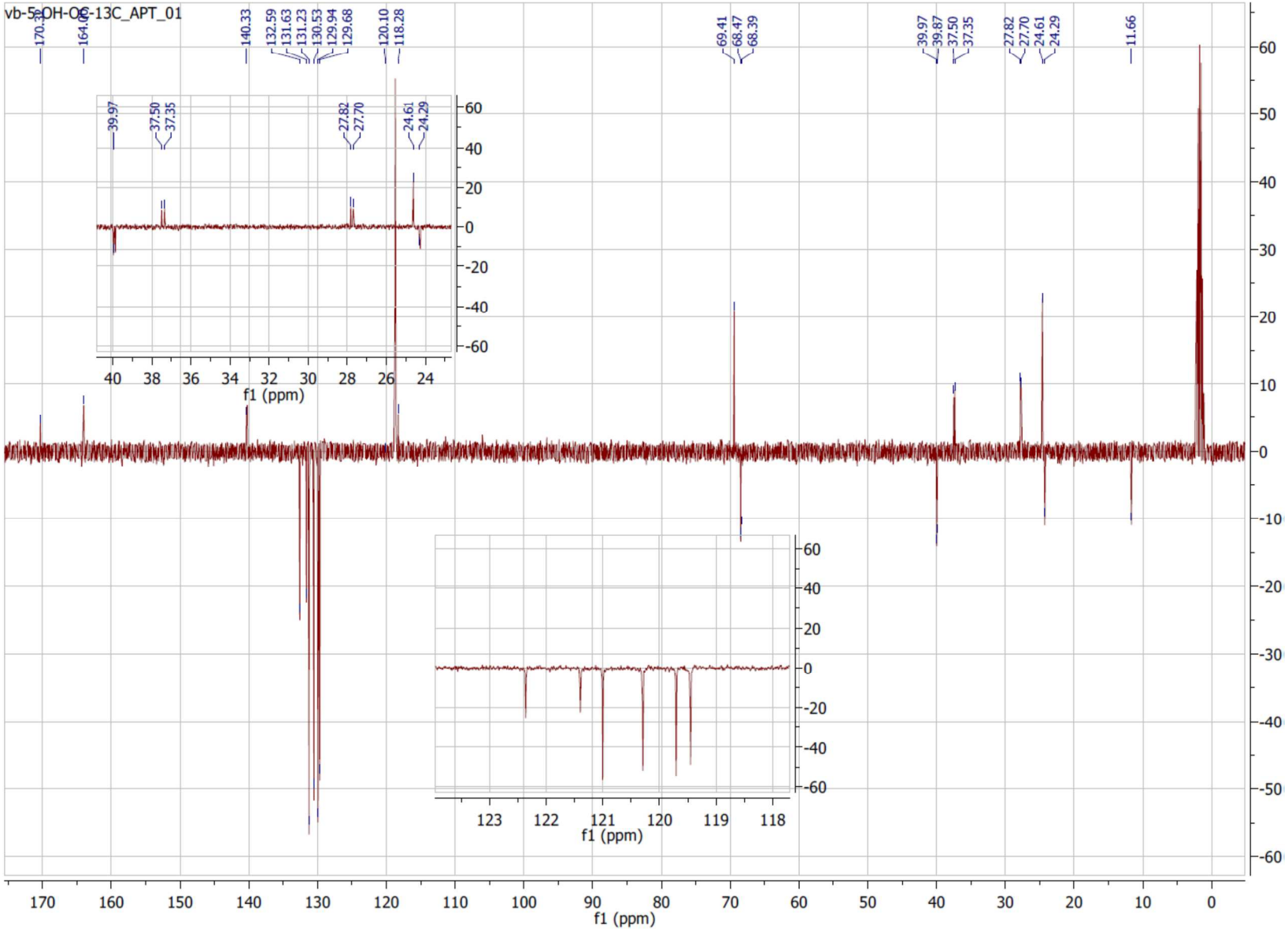


Figure S-4. ^{13}C-APT NMR spectrum of 5OH-OC

^{1}H NMR (400 MHz, Acetonitrile-d_3) δ 4.09 – 3.91 (m, 2H), 3.60 (ddt, J = 9.2, 6.2, 4.6 Hz, 1H), 1.48 – 1.37 (m, 1H), 1.36 – 1.24 (m, 3H), 1.24 – 1.13 (m, 2H), 1.09 (dt, J = 6.2, 0.7 Hz, 3H), 0.89 – 0.72 (m, 3H).

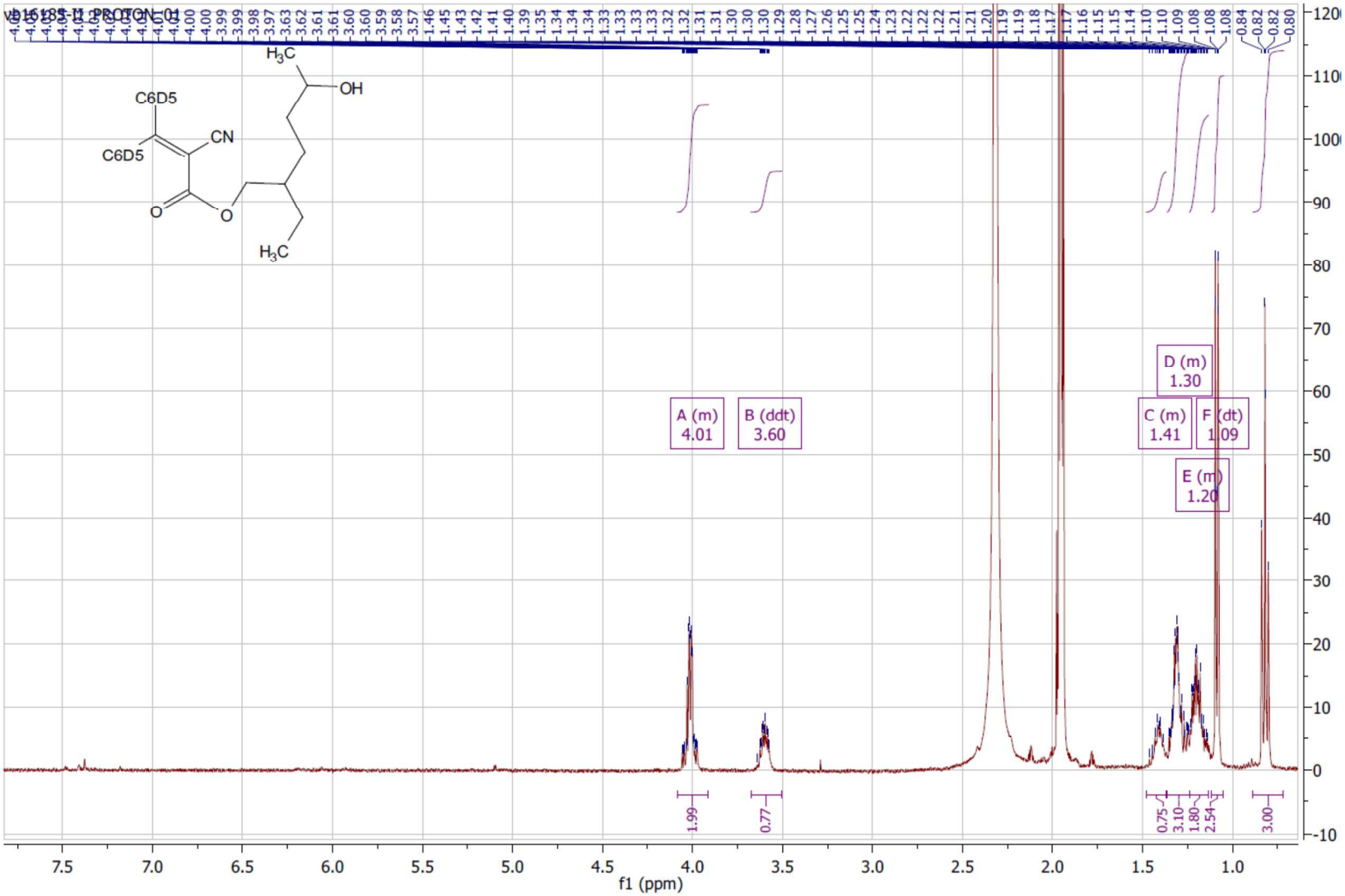


Figure S-5. ^{1}H NMR spectrum of 5OH-OC-d_{10}

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.41 (dd, *J* = 9.0, 7.4 Hz, 1H), 3.94 (dd, *J* = 9.0, 7.0 Hz, 1H), 2.62 (dd, *J* = 17.2, 8.4 Hz, 1H), 2.52 – 2.42 (m, 1H), 2.18 (dd, *J* = 17.2, 7.8 Hz, 1H), 1.51 (pd, *J* = 7.4, 3.1 Hz, 2H), 0.95 (t, *J* = 7.4 Hz, 3H).

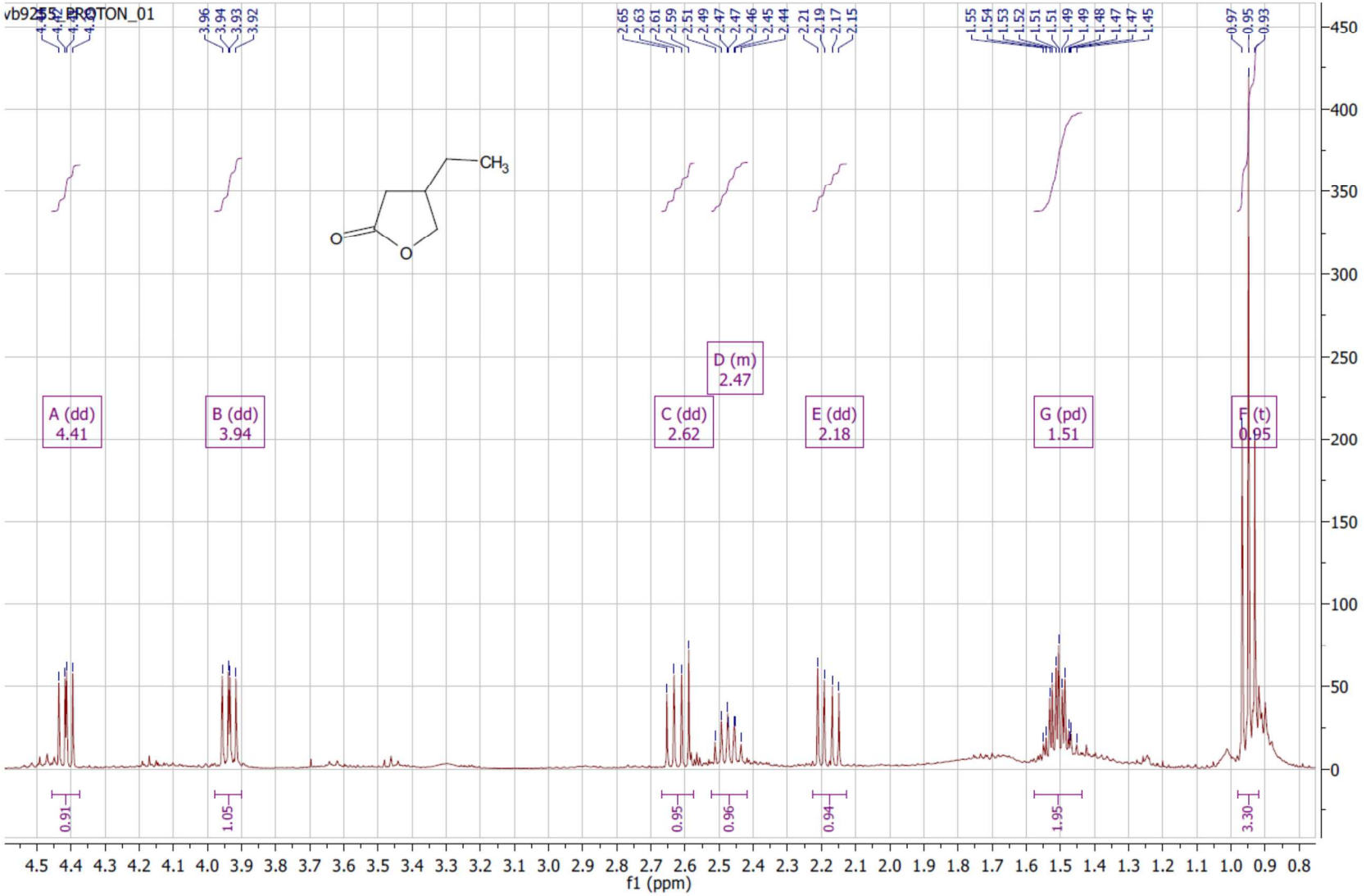


Figure S-6. ^{1}H NMR spectrum of dihydro-4-ethyl-2(3*H*)-furanone

^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.92 (ddt, *J* = 17.2, 10.4, 5.8 Hz, 1H), 5.32 (dq, *J* = 17.2, 1.5 Hz, 1H), 5.23 (dd, *J* = 10.4, 1.3 Hz, 1H), 4.58 (dt, *J* = 5.8, 1.4 Hz, 2H), 3.66 (dd, *J* = 10.9, 4.7 Hz, 1H), 3.52 (dd, *J* = 11.0, 6.7 Hz, 1H), 2.49 – 2.33 (m, 2H), 1.97 (dddd, *J* = 7.4, 6.7, 5.7, 4.6 Hz, 1H), 1.52 – 1.24 (m, 2H), 0.93 (t, *J* = 7.5 Hz, 3H).

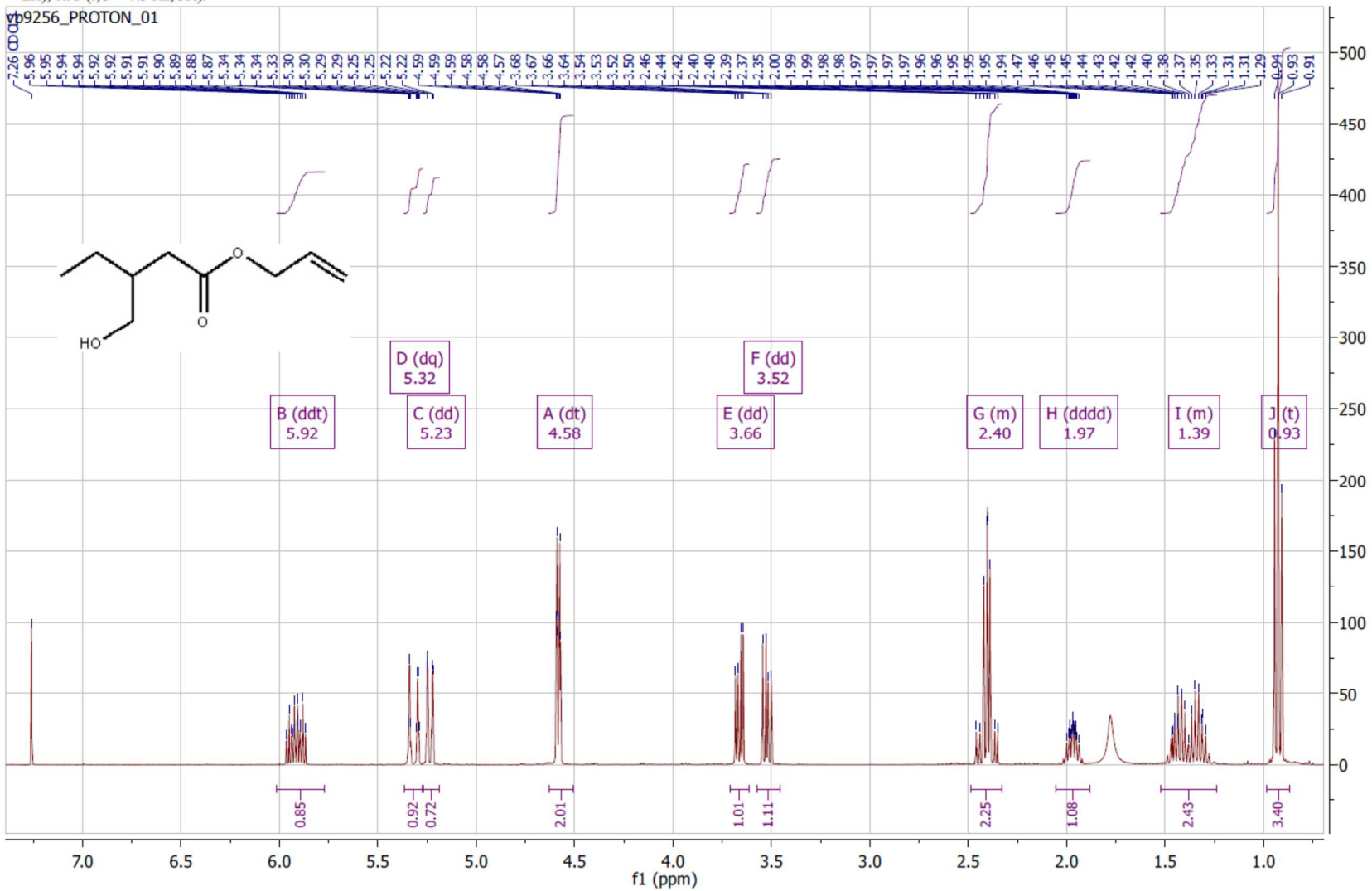


Figure S-7. ^{1}H NMR spectrum of allyl 3-(hydroxymethyl)pentanoate

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.54 – 7.31 (m, 8H), 7.21 – 7.08 (m, 2H), 4.15 (dd, *J* = 11.1, 4.7 Hz, 1H), 4.02 (dd, *J* = 11.1, 6.3 Hz, 1H), 2.20 (dd, *J* = 6.7, 0.8 Hz, 2H), 2.01 (qd, *J* = 6.7, 4.8 Hz, 1H), 1.38 – 1.21 (m, 2H), 0.87 (t, *J* = 7.5 Hz, 3H).

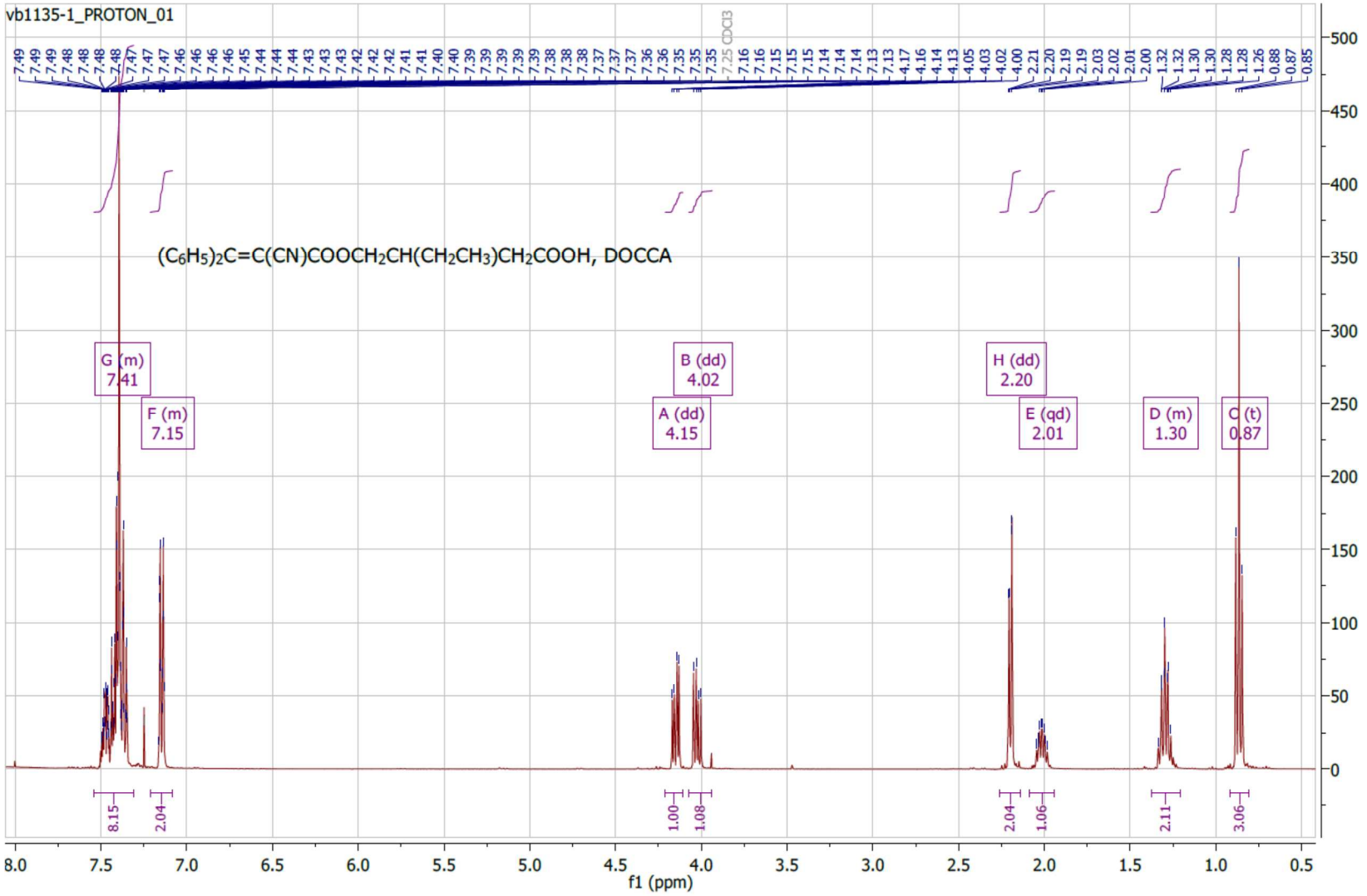


Figure S-8. ^{1}H NMR spectrum of DOCCA

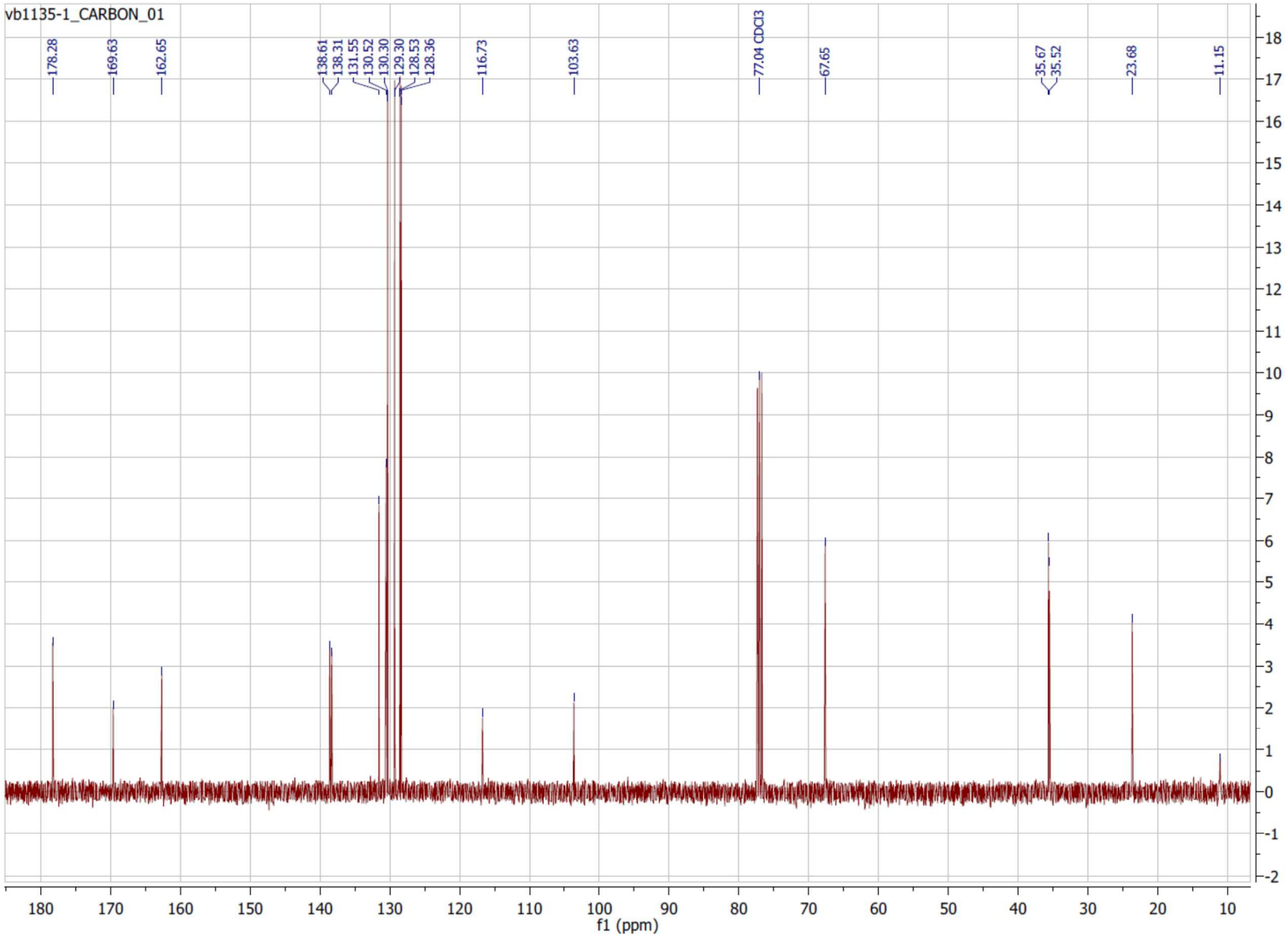


Figure S-9. ^{13}C NMR spectrum of DOCCA

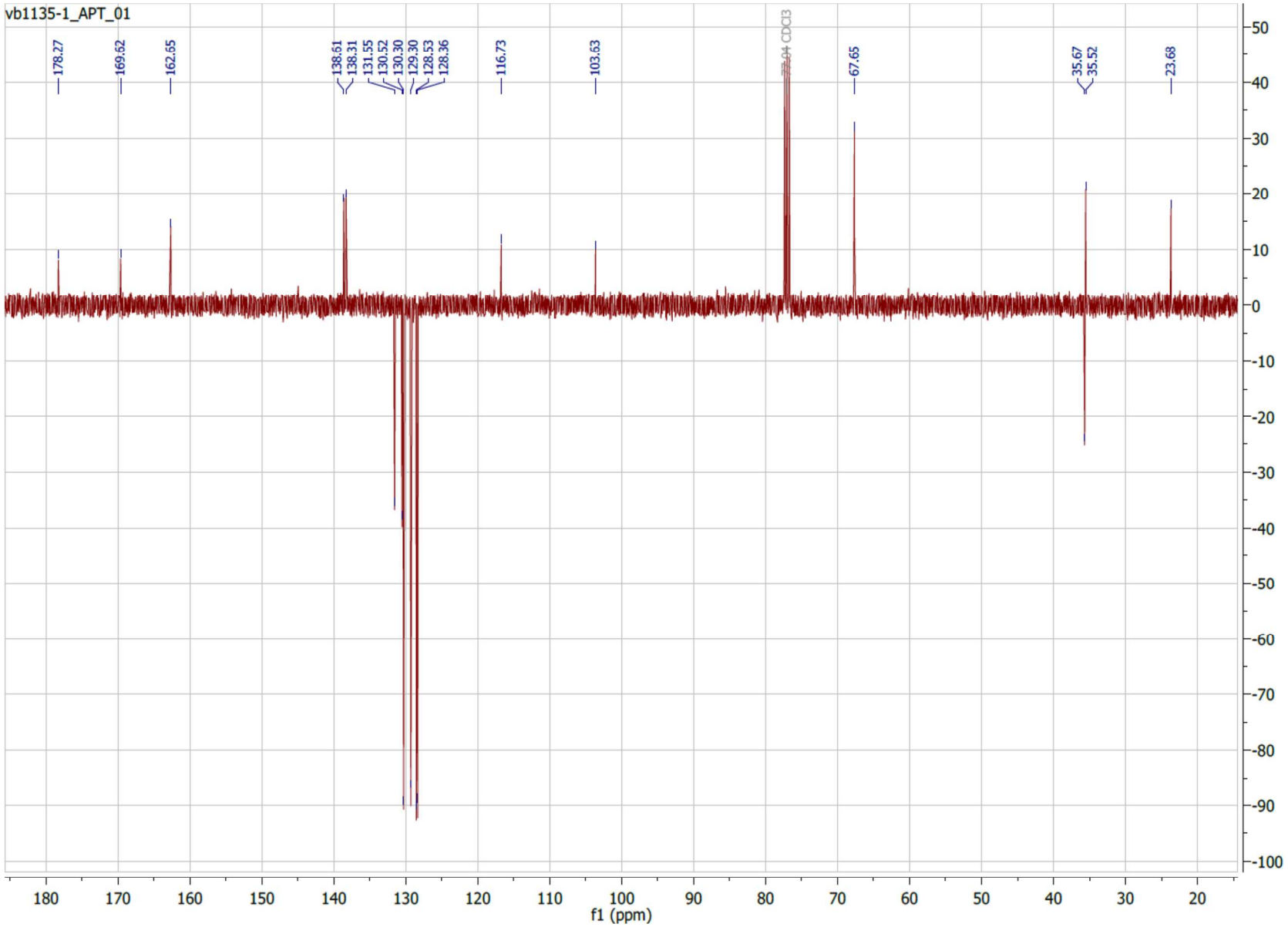


Figure S-10. ^{13}C-APT NMR spectrum of DOCCA

^{1}H NMR (400 MHz, Chloroform-d) δ 4.17 (dd, J = 11.1, 4.8 Hz, 1H), 4.03 (dd, J = 11.1, 6.4 Hz, 1H), 2.22 (dd, J = 6.8, 1.7 Hz, 2H), 2.03 (qd, J = 6.7, 4.7 Hz, 1H), 1.31 (qd, J = 7.8, 6.8 Hz, 2H), 0.88 (t, J = 7.5 Hz, 3H).

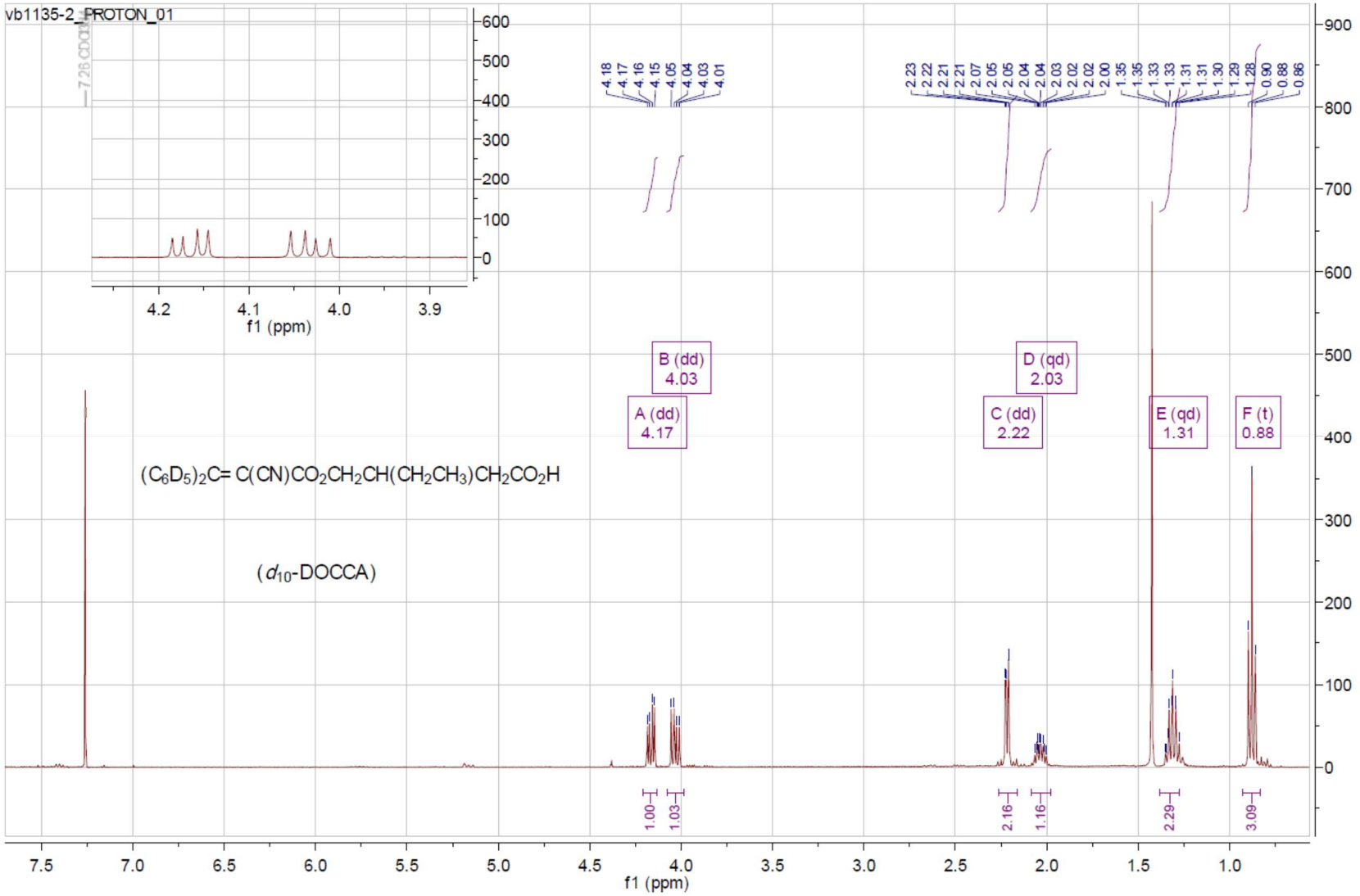


Figure S-11. ^{1}H NMR spectrum of DOCCA-d_{10}

Supporting information on "HR-MS and UHR-MS Product Ion Spectra"

HR-MS full scan spectra of all analytical standards and deuterium-labeled standards were recorded with LIT-Orbitrap-MS (LTQ Orbitrap XL, Thermo Scientific, Bremen, Germany) in ESI positive ion mode in a mass range of *m/z* 100 to 2000.

HR-MS product ion spectra of all analytical standards and deuterium-labeled standards were recorded with Q-Orbitrap-MS (Q Exactive Focus, Thermo Scientific, Bremen, Germany) at maximum resolution setting (R = 70,000). The following conditions were used: Spray voltage was 2.5 kV in negative (CPAA and CPAA-d_{10}) and 3.5 kV in positive (all standards) ion mode. Capillary and auxiliary gas heater temperatures were 250 °C and 100 °C, respectively. Sheath, auxiliary and sweep gas were set to 15, 5, and 0 arbitrary units. S-lens RF level was 50 arbitrary units.

For CPAA and CPAA-d_{10} UHR-MS spectra were recorded with FT-ICR-MS. Samples were ionized using electrospray ionization (ESI) in positive ion mode. Mass spectra were recorded using a Solarix 7 Tesla FTICR-MS instrument (Bruker, Bremen, Germany). In MS/MS mode, precursor ions were isolated first in the quadrupole and externally accumulated for 0.5 s, and then collision-induced dissociation (CID) was applied with collision voltage from 5-30 eV.

Supporting information on "Standard Solutions"

Approximately 5 mg of each deuterium-labeled standard were weighed exactly in a volumetric flask and dissolved and filled up to 5 mL with acetonitrile. Dilutions were prepared in acetonitrile (CPAA-d_{10}: 10 mg/L; DOCCA-d_{10}: 500 µg/L; 5OH-OC-d_{10}: 500 µg/L). These were used to prepare an internal standard mix (200 µg/L CPAA-d_{10}; 5 µg/L DOCCA-d_{10}; 1 µg/L 5OH-OC-d_{10}) in acetonitrile. Stock solutions of the non-labeled standards were prepared by exactly weighing

approximately 5 mg (CPAA) or 10 mg (DOCCA and 5OH-OC) of each non-labeled analytical standard in volumetric flasks and dissolving and filling up to a volume of 10 mL with acetonitrile. For DOCCA and 5OH-OC further dilutions (10 mg/L) in acetonitrile were prepared. From these solutions, three mixed analyte stock solutions were prepared in acetonitrile by serial dilution (a) 5.00 mg/L CPAA, 250 µg/L DOCCA and 5OH-OC; b) 250 µg/L CPAA, 12.5 µg/L DOCCA and 5OH-OC; c) 20.0 µg/L CPAA, 1.00 µg/L DOCCA and 5OH-OC). All solutions were stored in glass flasks, capped with screw caps with silicone/teflon seals at -20 °C. From the mixed analyte stock solutions, seven calibration solutions (0.2 to 100 µg/L CPAA; 10 ng/L to 5 µg/L DOCCA and 5OH-OC) were prepared in water. The calibration solutions, including water as a blank solution, were immediately aliquoted in HPLC vials and stored at -20°C until use. Calibration solutions were stable for at least four weeks.

Supporting information on "Chromatographic Conditions"

The quaternary pump ("loading pump") was in row with the autosampler, an in-line filter (0.5 µm porosity; 3.0 mm; Phenomenex, Aschaffenburg, Germany), and the 6-port switching valve. Flow from the autosampler was either directed via the switching valve onto the enrichment column and then into waste (during loading, analysis, and re-equilibration) or directly into waste (transfer step). The binary pump ("LC pump") was connected to the switching valve to deliver eluent flow either onto the enrichment column and then onto the HPLC column (transfer step) or directly onto the HPLC column (during loading, analysis, and re-equilibration). Gradients were as follows: The LC pump delivered eluent at a flow rate of 300 µL/min, starting at 30% B. After 2 min, B was increased to 50% within 2 min and then to 62% within 12 min. B was then increased to 95% within 2 min and kept for 5 min. Finally, B was returned to starting conditions within 0.5 min and kept for 8 min. The gradient for the loading pump is described in table S-1. Gradient delay

volumes according to the manufacturer's manuals are: 600 to 800 µL for the LC pump flow path, and 870 to 1170 µL for the loading pump flow path (600 to 900 µL for the quaternary pump and 270 µL for the autosampler). Enrichment column switching was performed at 3 min (start of analyte transfer) and 6 min (start of chromatographic separation). The thermostatted column compartment was kept at 25±1 °C. The injection volume was 100 µL. The autosampler was kept at 6 °C and the injection procedure included a needle wash with methanol/water 8:2 (v/v) for 10 s after sample aspiration.

Table S-1. Gradient for the loading pump

Time [min]	Flow rate [µL/min]	Eluent A [%]	Eluent B [%]
0	1500	100	0
3	1500	100	0
3.1	200	100	0
6	200	100	0
7	500	100	0
10	500	5	95
21	500	5	95
22	500	100	0
28.5	500	100	0
29.5	1500	100	0
31.5	1500	100	0

Supporting information to Results and Discussion

Estimation of LOQs

For the estimation of LOQs, a total of 187 individual native urine samples (urine samples from a human metabolism study – manuscript in preparation –, urine samples used during validation procedure, and spot urines from the pilot population) was analyzed. Metabolite peaks with signal-to-noise ratios (S/N) around or below 10 were integrated and metabolite concentrations were quantified. LOQ was the highest concentration with corresponding S/N of 10 (or slightly above). As an additional condition no concentration was higher in any sample with corresponding S/N<10. LOQs for all analytes were within the linear dynamic range of the calibration.

Sample preparation

To test the ruggedness of the deconjugation step, six urine samples with different native metabolite levels (CPAA: 45.1 to 1550 µg/L; DOCCA: 0.040 to 4.76 µg/L; 5OH-OC: <LOQ to 0.75 µg/L) were incubated for 1, 2, 3, 4, and 5 h. Also, samples were analyzed without addition of enzyme (negative control). Metabolite levels compared to the negative control were increased in all samples treated with enzyme. Approximate amounts of unconjugated metabolite (measured in the negative controls) were 90-100% for CPAA, <25% for DOCCA, and 0% for 5OH-OC. Maximum concentrations of the analytes, released from their respective conjugates, were reached after 1 h of incubation for all analytes in all urine samples, with longer incubation times having no further effect. Thus, incubation for 3 h was robust and should be unaffected by minor differences in enzymatic activity between enzyme batches.

Supporting information on "Mass spectrometry"

Figure S-12 shows the QqQ product ion spectra of all analytical standards, including deuterium-labeled standards. For accurate masses (including errors) of fragment ions observed in FT-ICR-MS and Q-Orbitrap-MS, see table S-2 to table S-9. In positive ion mode, all CPAA fragments except *m/z* 77 and all CPAA-d_{10} fragments except those corresponding to non-labeled fragments *m/z* 77, as well as *m/z* 206 and 208 corresponding to non-labeled fragment *m/z* 201 and *m/z* 210 corresponding to non-labeled fragment *m/z* 202, were observed in FT-ICR-MS (±0.14 ppm and better). The remaining fragments were verified by Q-Orbitrap-MS (±3.7 ppm), with exception of *m/z* 210 and 208 corresponding to non-labelled *m/z* 202 and 201, respectively, because mass resolution was too low for differentiation from isobaric fragments. For CPAA/CPAA-d_{10} in negative ion mode and 5OH-OC/5OH-OC-d_{10} and DOCCA/DOCCA-d_{10} in positive ion mode all fragments were verified by Q-Orbitrap-MS (±4.9 ppm and better). For CPAA the fragment at *m/z* 151 is most likely derived from a contamination; it was neither detected in FT-ICR-MS, nor in Q-Orbitrap-MS and there is no corresponding fragment for CPAA-d_{10}. The mass signals at *m/z* 354 (DOCCA) and 364 (DOCCA-d_{10}) were not observed in Q-Orbitrap-MS and cannot be explained. The mass difference of 10 u to $[M+H]^+$ makes it likely, that adduct formation (Na-adducts observed in full scan MS spectra) and/or DOCCA oligomers are involved. The mass signal *m/z* 272 for DOCCA is most likely derived from a contamination; it was not observed in Q-Orbitrap-MS and no corresponding mass signal for DOCCA-d_{10} was found.

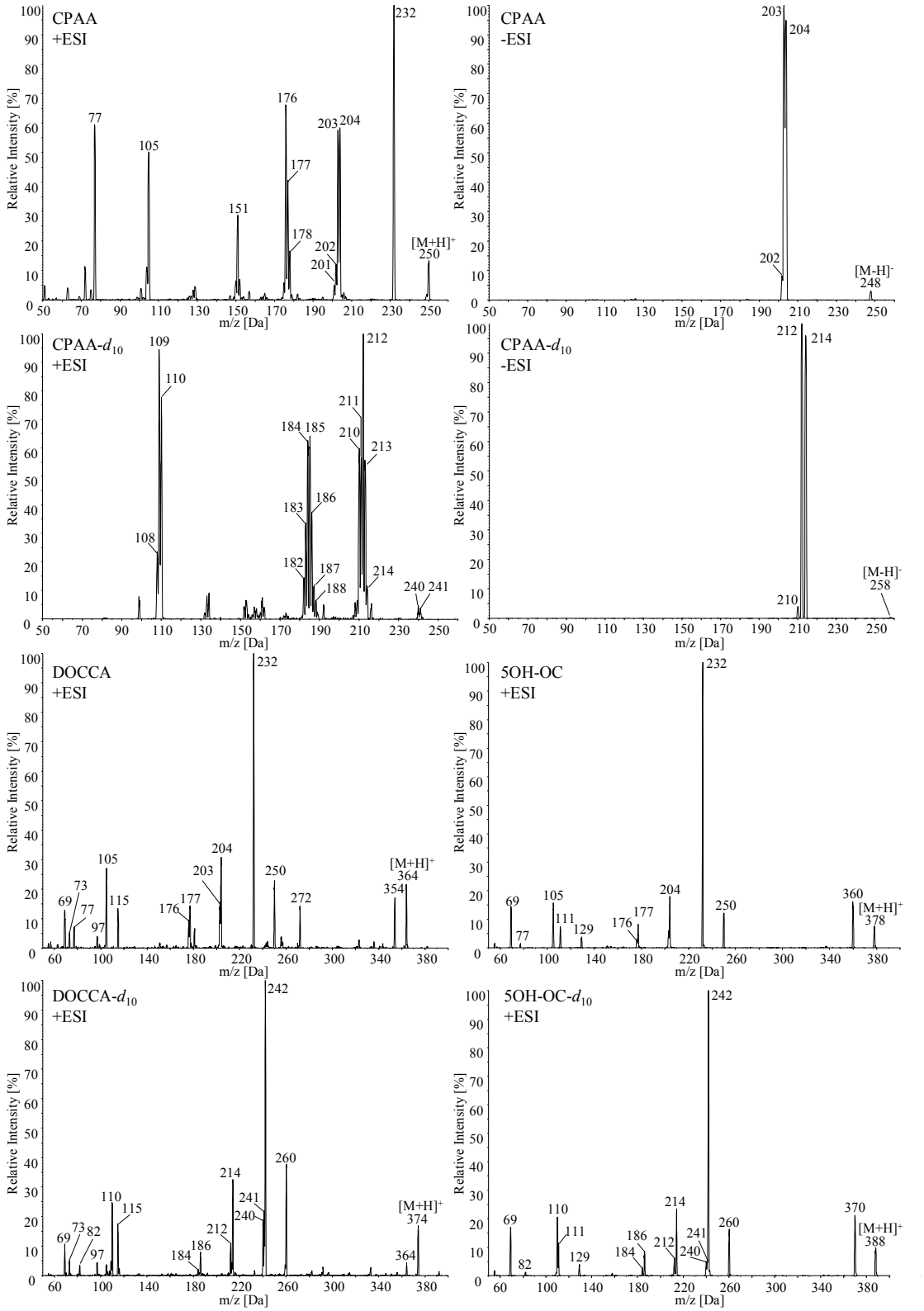


Figure S-12. QqQ-MS product ion spectra of non-labeled and deuterium-labeled standards in ESI positive ion mode (all standards) and negative ion mode (CPAA and CPAA-d_{10}).

Table S-2. Chemical formulae of postulated fragment structures, exact masses and accurate masses with errors (Q-Orbitrap-MS) for CPAA in negative ion mode

Chemical formula	Exact mass [*m/z*]	Accurate mass (error) [*m/z*]
$C_{15}H_{10}N^-$	204.08187	204.0820 (+0.6 ppm)
$C_{15}H_9N^{\bullet-}$	203.07405	203.0743 (+1.2 ppm)
$C_{15}H_8N^-$	202.06622	202.0665 (+1.4 ppm)

Table S-3. Chemical formulae of postulated fragment structures, exact masses and accurate masses with errors (Q-Orbitrap-MS) for CPAA-d_{10} in negative ion mode

Chemical formula	Exact mass [*m/z*]	Accurate mass (error) [*m/z*]
$C_{15}D_{10}N^-$	214,14464	214,1449 (+1.2 ppm)
$C_{15}D_9N^{\bullet-}$	212,13054	212,1308 (+1.2 ppm)
$C_{15}D_8N^-$	210,11644	210,1167 (+1.2 ppm)

Table S-4. Chemical formulae of postulated fragment structures, exact masses and accurate masses with errors (FT-ICR-MS and Q-Orbitrap-MS) for CPAA in positive ion mode

Chemical formula	Exact mass [*m/z*]	Accurate mass (error) [*m/z*]	
		FT-ICR-MS	Q-Orbitrap-MS
$C_{16}H_{10}NO^+$	232.07569	232.07567 (-0.09 ppm)	232.0751 (-2.5 ppm)
$C_{15}H_{10}N^+$	204.08078	204.08076 (-0.10 ppm)	204.0803 (-2.4 ppm)
$C_{15}H_9N^{\bullet+}$	203.07295	203.07293 (-0.10 ppm)	203.0725 (-2.2 ppm)
$C_{15}H_8N^+$	202.06513	202.06512 (-0.05 ppm)	202.0648 (-1.6 ppm)
$C_{15}H_7N^{\bullet+}$	201.05730	201.05732 (-0.10 ppm)	201.0569 (-2.0 ppm)
$C_{14}H_{10}^{\bullet+}$	178.07770	178.07771 (-0.06 ppm)	178.0773 (-2.3 ppm)
$C_{14}H_9^+$	177.06988	177.06988 (±0.00 ppm)	177.0695 (-2.2 ppm)
$C_{14}H_8^{\bullet+}$	176.06205	176.06205 (±0.00 ppm)	176.0617 (-2.0 ppm)
$C_7H_5O^+$	105.03349	105.03349 (±0.00 ppm)	105.0333 (-1.8 ppm)
$C_6H_5^+$	77.03858	- [a]	77.0385 (-1.0 ppm)

[a]: not observed

Table S-5. Chemical formulae of postulated fragment structures, exact masses and accurate masses with errors (FT-ICR-MS and Q-Orbitrap-MS) for CPAA-d_{10} in positive ion mode

Chemical formula	Exact mass [*m/z*]	Accurate mass (error) [*m/z*]	
		FT-ICR-MS	Q-Orbitrap-MS
$C_{16}D_{10}NO^+$	242.13846	242.13845 (-0.04 ppm)	-[b]
$^{13}C_1C_{15}HD_9NO^+$	242.13554	242.13556 (+0.08 ppm)	-[b]
$C_{16}HD_9NO^+$	241.13218	241.13218 (±0.00 ppm)	-[b]
$^{13}C_1C_{15}H_2D_8NO^+$	241.12926	241.12925 (-0.04 ppm)	-[b]
$C_{16}H_2D_8NO^+$	240.12590	240.12591 (+0.04 ppm)	-[b]
$^{13}C_1C_{15}H_3D_7NO^+$	240.12298	240.12296 (-0.08 ppm)	-[b]
$C_{15}D_{10}N^+$	214.14354	214.14354 (±0.00 ppm)	214.1431 (-2.1 ppm)
$^{13}C_1C_{14}HD_9N^+$	214.14062	214.14064 (0.09 ppm)	-[a]
$C_{15}HD_9N^+$	213.13727	213.13726 (-0.05 ppm)	213.1366 (-3.1 ppm)
$^{13}C_1C_{14}H_2D_8N^+$	213.13434	213.13433 (-0.05 ppm)	-[a]
$C_{15}H_2D_8N^+$	212.13099	212.13098 (-0.05 ppm)	-[b]
$C_{15}D_9N^{\bullet+}$	212.12944	212.12944 (±0.00 ppm)	-[b]
$^{13}C_1C_{14}HD_8N^{\bullet+}$	212.12652	212.12652 (±0.00 ppm)	-[a]
$C_{15}HD_8N^{\bullet+}$	211.12316	211.12317 (+0.05 ppm)	211.1228 (-1.7 ppm)
$^{13}C_1C_{14}H_2D_7N^{\bullet+}$	211.12024	211.12026 (+0.09 ppm)	-[a]
$C_{15}H_2D_7N^{\bullet+}$	210.11689	210.11688 (-0.05 ppm)	-[b]
$C_{15}D_8N^+$	210.11534	-[a]	-[b]
$C_{15}HD_7N^+$	209.10906	209.10907 (+0.05 ppm)	209.1087 (-1.7 ppm)
$C_{15}H_2D_6N^+$	208.10279	208.10276 (-0.14 ppm)	-[b]
$C_{15}D_7N^{\bullet+}$	208.10124	-[a]	-[b]
$C_{15}HD_6N^{\bullet+}$	207.09496	207.09494 (-0.1 ppm)	207.0947 (-1.3 ppm)
$C_{15}H_2D_5N^{\bullet+}$	206.08868	-[a]	206.0883 (-1.8 ppm)
$C_{14}D_{10}^{\bullet+}$	188.14047	188.14048 (+0.05 ppm)	188.1404 (-0.4 ppm)
$^{13}C_1C_{13}HD_9^{\bullet+}$	188.13755	188.13757 (+0.11 ppm)	-[a]
$C_{14}HD_9^{\bullet+}$	187.13419	187.13419 (±0.00 ppm)	187.1338 (-2.1 ppm)
$^{13}C_1C_{13}H_2D_8^{\bullet+}$	187.13127	187.13128 (+0.05 ppm)	-[a]
$C_{14}H_2D_8^{\bullet+}$	186.12792	186.12792 (±0.00 ppm)	-[b]
$C_{14}D_9^+$	186.12637	186.12635 (-0.11 ppm)	-[b]
$^{13}C_1C_{13}HD_8^+$	186.12345	186.12345 (±0.00 ppm)	-[a]
$C_{14}HD_8^+$	185.12009	185.12009 (±0.00 ppm)	185.1197 (-2.1 ppm)
$^{13}C_1C_{13}H_2D_7^+$	185.11717	185.11718 (+0.05 ppm)	-[a]
$C_{14}H_2D_7^+$	184.11381	184.11381 (±0.00 ppm)	-[b]
$C_{14}D_8^{\bullet+}$	184.11227	184.11227 (±0.00 ppm)	-[b]
$^{13}C_1C_{13}HD_7^{\bullet+}$	184.10934	184.10934 (±0.00 ppm)	-[a]
$C_{14}HD_7^{\bullet+}$	183.10599	183.10598 (-0.05 ppm)	183.1056 (-2.1 ppm)
$^{13}C_1C_{13}H_2D_6^{\bullet+}$	183.10307	183.10309 (+0.11 ppm)	-[a]
$C_{14}H_2D_6^{\bullet+}$	182.09971	182.09971 (±0.00 ppm)	182.0992 (-2.8 ppm)

[a]: not observed; [b]: resolution too low

Table S-5 - continued. Chemical formulae of postulated fragment structures, exact masses and accurate masses with errors (FT-ICR-MS and Q-Orbitrap-MS) for CPAA-d_{10} in positive ion mode

Chemical formula	Exact mass [*m/z*]	Accurate mass (error) [*m/z*]	
		FT-ICR-MS	Q-Orbitrap-MS
$^{13}C_1C_6D_5O^+$	111.06823	111.06823 (±0.00 ppm)	-[a]
$C_7D_5O^+$	110.06487	110.06488 (+0.09 ppm)	110.0646 (-2.5 ppm)
$C_7HD_4O^+$	109.05860	109.05860 (±0.00 ppm)	109.0584 (-1.8 ppm)
$C_7H_2D_3O^+$	108.05232	108.05232 (±0.00 ppm)	108.0520 (-3.0 ppm)
$C_6D_5^+$	82.06996	-[a]	82.0699 (-0.7 ppm)
$C_6HD_4^+$	81.06368	-[a]	81.0636 (-1.0 ppm)
$C_6H_2D_3^+$	80.05741	-[a]	80.0573 (-1.4 ppm)

[a]: not observed; [b]: resolution too low

Table S-6. Chemical formulae of postulated fragment structures, exact masses and accurate masses with errors (Q-Orbitrap-MS) for DOCCA in positive ion mode

Chemical formula	Exact mass [*m/z*]	Accurate mass (error) [*m/z*]
$C_{16}H_{12}NO_2^+$	250.08626	250.0855 (-3.0 ppm)
$C_{16}H_{10}NO^+$	232.07569	232.0749 (-3.4 ppm)
$C_{15}H_{10}N^+$	204.08078	204.0802 (-2.8 ppm)
$C_{15}H_9N^{\bullet+}$	203.07295	203.0724 (-2.7 ppm)
$C_{15}H_8N^+$	202.06513	202.0646 (-2.6 ppm)
$C_{14}H_{10}^{\bullet+}$	178.07770	178.0772 (-2.8 ppm)
$C_{14}H_9^+$	177.06988	177.0694 (-2.7 ppm)
$C_{14}H_8^{\bullet+}$	176.06205	176.0616 (-2.6 ppm)
$C_6H_{11}O_2^+$	115.07536	115.0751 (-2.3 ppm)
$C_7H_5O^+$	105.03349	105.0332 (-2.8 ppm)
$C_6H_9O^+$	97.06479	97.0646 (-2.0 ppm)
$C_6H_5^+$	77.03858	77.0382 (-4.9 ppm)
$C_4H_9O^+$	73.06479	73.0647 (-1.2 ppm)
$C_5H_9^+$	69.06988	69.0698 (-1.2 ppm)

Table S-7. Chemical formulae of postulated fragment structures, exact masses and accurate masses with errors (Q-Orbitrap-MS) for DOCCA-d_{10} in positive ion mode

Chemical formula	Exact mass [*m/z*]	Accurate mass (error) [*m/z*]
$C_{16}H_2D_{10}NO_2^+$	260.14902	260.1482 (-3.2 ppm)
$C_{16}D_{10}NO^+$	242.13846	242.1376 (-3.6 ppm)
$C_{16}HD_9NO^+$	241.13218	241.1313 (-3.6 ppm)
$C_{16}H_2D_8NO^+$	240.12590	240.1251 (-3.3 ppm)
$C_{15}D_{10}N^+$	214.14354	214.1429 (-3.0 ppm)
$C_{15}HD_9N^+$	213.13727	213.1366 (-3.1 ppm)
$C_{15}H_2D_8N^+$	212.13099	-[b]
$C_{15}D_9N^{\bullet+}$	212.12944	-[b]
$C_{15}HD_8N^{\bullet+}$	211.12316	211.1228 (-1.7 ppm)
$C_{14}H_2D_8^{\bullet+}$	186.12792	-[b]
$C_{14}D_9^+$	186.12637	-[b]
$C_6H_{11}O_2^+$	115.07536	115.0751 (-2.3 ppm)
$C_7D_5O^+$	110.06487	110.0646 (-2.5 ppm)
$C_7HD_4O^+$	109.0586	109.0583 (-2.8 ppm)
$C_7H_2D_3O^+$	108.05232	108.052 (-3.0 ppm)
$C_6H_9O^+$	97.06479	97.0646 (-2.0 ppm)
$C_6D_5^+$	82.06996	82.0696 (-4.4 ppm)
$C_4H_9O^+$	73.06479	73.0647 (-1.2 ppm)
$C_5H_9^+$	69.06988	69.0698 (-1.2 ppm)

[b]: resolution too low

Table S-8. Chemical formulae of postulated fragment structures, exact masses and accurate masses with errors (Q-Orbitrap-MS) for 5OH-OC in positive ion mode

Chemical formula	Exact mass [*m/z*]	Accurate mass (error) [*m/z*]
$C_{24}H_{26}NO_2^+$	360.19581	360.1950 (-2.2 ppm)
$C_{16}H_{12}NO_2^+$	250.08626	250.0857 (-2.2 ppm)
$C_{16}H_{10}NO^+$	232.07569	232.0752 (-2.1 ppm)
$C_{15}H_{10}N^+$	204.08078	204.0804 (-1.9 ppm)
$C_{15}H_9N^{\bullet+}$	203.07295	203.0725 (-2.2 ppm)
$C_{15}H_8N^+$	202.06513	202.0648 (-1.6 ppm)
$C_{15}H_7N^{\bullet+}$	201.05730	201.0569 (-2.0 ppm)
$C_{14}H_{10}^{\bullet+}$	178.07770	178.0774 (-1.7 ppm)
$C_{14}H_9^+$	177.06988	177.0696 (-1.6 ppm)
$C_{14}H_8^{\bullet+}$	176.06205	176.0617 (-2.0 ppm)
$C_8H_{17}O^+$	129.12739	129.1272 (-1.5 ppm)
$C_8H_{15}^+$	111.11683	111.1167 (-1.2 ppm)
$C_7H_5O^+$	105.03349	105.0334 (-0.9 ppm)
$C_6H_5^+$	77.03858	77.0385 (-1.0 ppm)
$C_5H_9^+$	69.06988	69.0699 (+0.3 ppm)

Table S-9. Chemical formulae of postulated fragment structures, exact masses and accurate masses with errors (Q-Orbitrap-MS) for 5OH-OC-d_{10} in positive ion mode

Chemical formula	Exact mass [*m/z*]	Accurate mass (error) [*m/z*]
$C_{24}H_{16}D_{10}NO_2^+$	370.25857	370.2578 (-2.1 ppm)
$C_{16}H_2D_{10}NO_2^+$	260.14902	260.1484 (-2.4 ppm)
$C_{16}D_{10}NO^+$	242.13846	242.1377 (-3.1 ppm)
$C_{16}HD_9NO^+$	241.13218	241.1319 (-1.2 ppm)
$C_{16}H_2D_8NO^+$	240.12590	240.1253 (-2.5 ppm)
$C_{15}D_{10}N^+$	214.14354	214.1431 (-2.1 ppm)
$C_{15}HD_9N^+$	213.13727	213.1369 (-1.7 ppm)
$C_{15}H_2D_8N^+$	212.13099	-[b]
$C_{15}D_9N^{\bullet+}$	212.12944	-[b]
$C_{15}HD_8N^{\bullet+}$	211.12316	211.1229 (-1.2 ppm)
$C_{14}D_{10}^{\bullet+}$	188.14047	188.1402 (-1.4 ppm)
$C_{14}HD_9^{\bullet+}$	187.13419	187.1339 (-1.5 ppm)
$C_{14}H_2D_8^{\bullet+}$	186.12792	-[b]
$C_{14}D_9^+$	186.12637	-[b]
$C_{14}HD_8^+$	185.12009	185.1198 (-1.6 ppm)
$C_{14}H_2D_7^+$	184.11381	-[b]
$C_{14}D_8^{\bullet+}$	184.11227	-[b]
$C_{14}HD_7^{\bullet+}$	183.10599	183.1058 (-1.0 ppm)
$C_{14}H_2D_6^{\bullet+}$	182.09971	182.0996 (-0.6 ppm)
$C_8H_{17}O^+$	129.12739	129.1272 (-1.5 ppm)
$C_8H_{15}^+$	111.11683	111.1167 (-1.2 ppm)
$C_7D_5O^+$	110.06487	110.0648 (-0.6 ppm)
$C_7HD_4O^+$	109.0586	109.0585 (-0.9 ppm)
$C_7H_2D_3O^+$	108.05232	108.0522 (-1.1 ppm)
$C_6D_5^+$	82.06996	82.0699 (-0.7 ppm)
$C_6HD_4^+$	81.06368	81.0636 (-1.0 ppm)
$C_6H_2D_3^+$	80.05741	80.0573 (-1.4 ppm)
$C_5H_9^+$	69.06988	69.0699 (+0.3 ppm)

[b]: resolution too low

Exemplary calibration curves

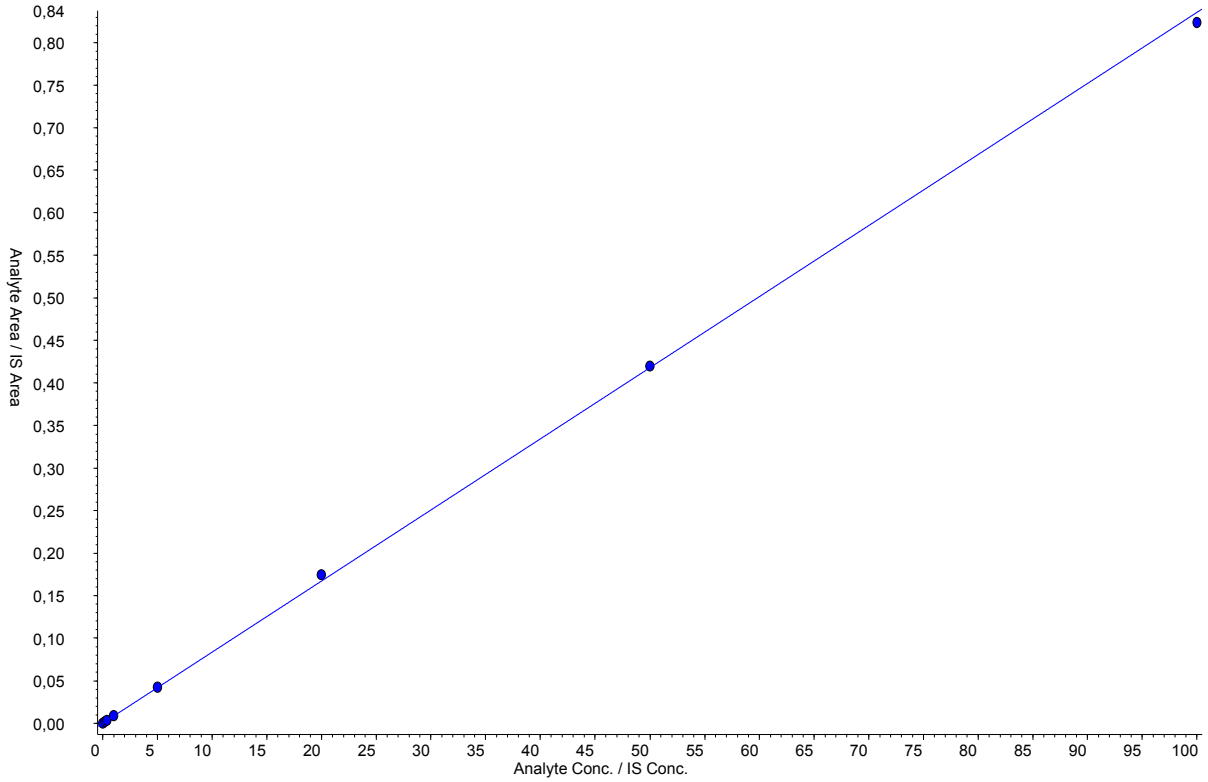


Figure S-13. Exemplary calibration curve for CPAA (r = 0.9998)

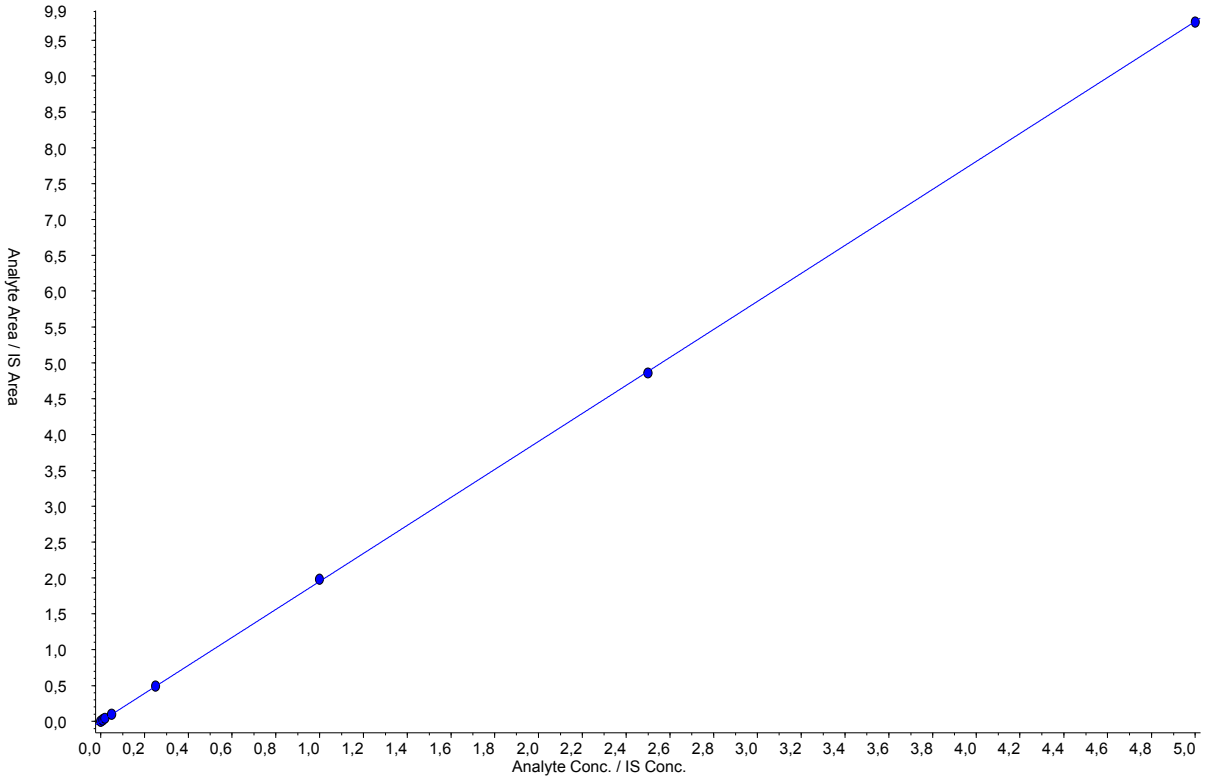


Figure S-14. Exemplary calibration curve for DOCCA (r = 1.0000)

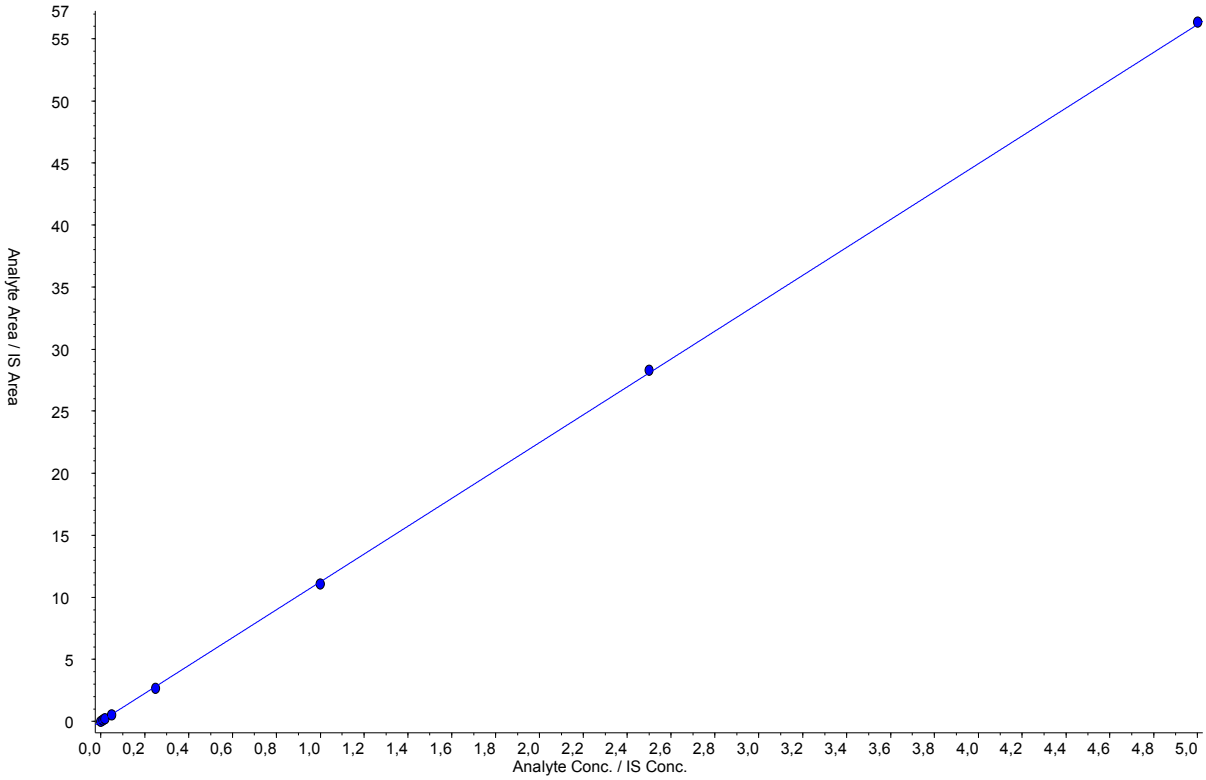


Figure S-15. Exemplary calibration curve for 5OH-OC (r = 0.9999)